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Our study proposes to test the hypothesis that ionizing radiation can transform to a malignant phenotype immortalized/initiated breast cells from a donor with Li-Fraumeni syndrome (LFS) containing a germline mutation. Human breast cells were irradiated using a protocol similar to that used in the therapeutic treatment of breast carcinoma in situ. Exponentially growing cells were inoculated at 5 x 10^5 per 75cm flask and irradiated 24hrs later with 2Gy of gamma radiation at a dose rate of 2.37Gy/min. Additional doses of 2Gy were delivered at daily intervals to a total dose of 60Gy. After each 10Gy increment, the cultures were allowed to recover for 7-10 days. After this recovery, a portion of the irradiated cultures were tested for changes in morphology, anchorage-independent growth, growth factor requirements (i.e. removal of BPE, EGF or insulin), growth in presence of serum and tumorigenicity. Potential transformants were tested for the development of the anchorage-independent phenotype following radiation treatment by suspension in 0.3% agar at a concentration of 1x10^4/ml and cells were examined for clonal growth at 21 days. In addition, three-week old female SCID mice were inoculated in the abdominal mammary fatpad with 10^7 unirradiated or radiation-treated human breast cells.

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Peter J Yhraves 7/30/97

PI - Signature Date

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INTRODUCTION

The complexity of human breast cancer and the development of 120,000 new cases annually contrasts with our relative lack of knowledge of the biology of the disease (1). An improved understanding of this complex disease would be helped by determining the environmental agents involved in its development. Epidemiological studies have shown a role for a combination of factors, hormonal/reproductive history, diet, socioeconomic status, genetic predisposition, and external factors such as ionizing radiation. Knowledge of the effects of ionizing radiation on the human mammary gland is important both for the estimation of environmental risk to human populations and for the study of normal tissues damage in the therapy of breast cancer. Although many epidemiological studies have shown a role for ionizing radiation in the development of human breast cancer, this issue has remained unresolved. Ionizing radiations under appropriate conditions will induce cancers in experimental animals and humans and can act as complete carcinogens since they can both initiate and promote neoplastic transformation (2,3). This carcinogenic potential has proven controversial regarding diagnostic techniques and a potential deterrent to its use in radiation therapy. However, the cancer incidences of the atomic bomb survivors (4-6), and other studies of North American women exposed to medical irradiation (7-9), show that the breast is one of the most sensitive human tissues for radiation-induced carcinogenesis (10).

The recent cloning of the Ataxia telangiectasia gene has renewed interest in individuals with potential genetic predispositions for breast cancer (11). Individuals who are heterozygous for ataxia telangiectasia (-1% of the population) exhibit two significant characteristics: cancer predisposition and radiation sensitivity. Individuals who are homozygous for ataxia telangiectasia have an exceptionally high incidence of all cancers (12) and those who are heterozygous for this condition have an excess risk of cancer, particularly breast cancer and are considered a susceptible population (13). Cancer predisposition in this group has been estimated to be about three- to fourfold that of the general population, with a relative risk for breast cancer in carriers fivefold that of normal women (14-20). Among women in families affected with the hereditary disease Li-Fraumeni syndrome (LFS), breast tumors are the most prevalent cancer (affecting at least 50%) with 28% of the breast cancers being diagnosed before age 30 and 89% before age 50 (21-23). In spite of these observations ionizing radiation is routinely used in the therapy of primary breast cancer with many early stage tumors being treated conservatively by the surgical removal of the tumor followed by treatment of the remaining breast and associated tissues with radiation (17). The biological effects of such therapeutic doses of ionizing radiation at or near the location of surgery have not been fully established. As the use of this type of treatment increases, there will be an increasing incidence of radiation exposure of normal and benign breast tissues in patients (18). With the occurrence of new solid tumors being a well-recognized consequence of therapeutic radiation (19,20), it will be important to determine the cellular and molecular effects of ionizing radiation on human breast cells.

Although ionizing radiations were one of the first proven environmental carcinogens (24,25), the molecular mechanisms of radiation carcinogenesis have remained poorly understood. Extensive studies using rodent cells in culture have developed quantitative relationships between dose, dose-rate, and quality of ionizing radiation with the eventual development of a tumorigenic

phenotype (26-36). Initial molecular studies of radiation carcinogenesis have described the activation of cellular *ras* genes in rodent experimental systems (37). However, there are fewer studies describing the radiation-induced transformation of human cells and the molecular mechanisms involved (38-40). While rodent model systems employed in mammary cancer research have clarified certain areas of investigation, the known differences between human and rodent mammary physiology, in response to etiological agents, emphasizes the uncertainty in applying information gained in these model systems to the human situation. The direct study of human cells is the most appropriate way to determine the potential of etiological agents to initiate and promote human mammary neoplasia.

There are now several immortalized human breast cells from individual donors displaying a variety of changes which correlate with those observed during neoplastic development, e.g.,extended lifespan, immortality, growth factor independence and tumorigenicity. While any of these immortalized cell lines cannot be considered to represent a normal phenotype, such cell lines with an indefinite lifespan are usually more amenable to experimentation than normal finite lifespan cells in determining the potential of chemical, physical carcinogens, oncogenic viruses or transfected genes, to induce malignant phenotypes.

The study of radiation-induced transformation of human cells has been hampered until the recent development of immortalized cell lines. Ionizing radiation as x-rays has been shown to extend the lifespan and immortalize normal embryonic human fibroblasts and epithelial cells (41-45). The development of a human epidermal keratinocyte cell line (RHEK) has provided a model system for studying human epithelial cell transformation (46,47). We have recently shown that these immortalized human keratinocytes can be transformed with x-rays into malignant cells (38). In a subsequent study we demonstrated that this radiation-induced transformation did not involve mutations or allelic losses in either the p53 tumor suppressor gene or the cellular *ras* genes (48). These studies and those performed by others used cells that were immortalized with viral oncogenes (SV40-T antigen or HPV16/18). Although such cells have proven useful in demonstrating malignant transformation of human cells with ionizing radiation, the presence of viral oncoprotein has made it difficult to evaluate the role of p53 tumor suppressor gene in radiation-induced malignant transformation of human cells. The availability of immortalized human breast epithelial cells not containing viral oncoproteins provide in vitro models to evaluate the role of p53. In a recent study by Wazer et.al. it was shown that normal human mammary epithelial cells can be transformed with 30Gray of gamma-radiation to produce malignant cells (40). The amount of radiation used in this study was within the range of that used during conventional radiotherapy of breast cancer, typically 2Gy daily fractions to a cumulative dose of 60-70Gy.This malignant transformation was accompanied by the complete loss of p53 protein expression due to deletions in both alleles of the p53 gene. This study demonstrates the potential of the p53 gene as a target for radiation-induced mutations in human breast epithelial cells. Alterations in the p53 gene are commonly found in several types of human neoplasms including breast cancer (49,50) and in patients with tumors purportedly caused by radiation therapy (51). Further, patients with Li-Fraumeni syndrome who have heritable alterations in p53 are at an increased risk of developing certain cancers, including breast cancer, after radiation exposure (52). In transgenic mice,deletions in the p53 gene result in an increased sensitivity to radiation-induced tumorigenesis (53). These observations strongly implicate p53 as an important determinant in radiation carcinogenesis.

Ionizing radiation is known to induce DNA double-strand breaks which can lead to chromosomal deletions and rearrangements (54,55). Structural changes associated with the induction of mutations by ionizing radiation at autosomal loci in human cells, indicate that more than 70% of x-ray induced mutations involve the entire loss of an entire gene (56). Deletions in regions of DNA, indicating the loss of a tumor suppressor gene are also relatively common in breast cancer, usually being detected as a loss of heterozygosity in a polymorphic allele. Allelic loss for chromosome 13 has also been found in approximately 25% of breast carcinomas (57) which is the location of the chromosomal region containing the retinoblastoma-susceptibility gene. The highest frequency of allelic loss in primary breast carcinomas has been found for a region on the short arm of chromosome 17 (58). This region includes the *p53*gene.

The p53 gene is a nuclear phosphoprotein which has been implicated in the control of normal proliferation and neoplastic transformation of cells (59,60). It is expressed at low levels in non-transformed cells but is often elevated in tumor-derived or transformed cell lines (60). Early studies showed that the p53 gene could function as a dominant transforming oncogene (61-63), however, these studies employed mutated p53 genes (60), and it has been shown that the wild-type gene is incapable of transformation (64-66). Further, expression of the wild type gene inhibits the activity of transforming genes in transfection assays (67), demonstrating that wild type p53 is a suppressor of cellular growth. Evidence has been obtained for the functional suppression of the cellular growth of several different human cancer cell lines following DNA transfection or retro viral transfer of the wild-type p53 gene (68-70). There is evidence that inactivating point mutations in the p53 gene are involved in the etiology of many human cancers (71-76). Evidence is accumulating that mutations in the p53 gene are important in the development of human breast cancer (72,77,78). Allelic losses have frequently been observed in the short arm of chromosome 17 in human breast tumors (79-81), consistent with the location of a tumor suppressor gene in this region. Although there may be more than one region of allelic loss on chromosome 17p (82), one is known to include the p53 gene at 17p13 (82-85). Frequent over-expression of the p53 gene has been reported in breast tumors (86) and there is a high correlation between elevated expression of the p53 gene and loss of heterozygosity on the short arm of chromosome 17 (87). Point mutations in the p53 gene have been detected in both breast cancer cell lines and primary tumors (72,77,78,88,90) and abnormal histochemical staining using p53 antibodies has been reported in approximately 50%of breast tumors examined (74,76-78,89).

Recently, two studies have shown germ line p53 mutations in fibroblasts derived from both affected and non-symptomatic individuals exhibiting the hereditary cancer disease Li-Fraumeni syndrome (21,90). Patients with this syndrome can develop variety of soft-tissue cancers, and breast cancer at an early age (22). A molecular explanation for the specifically increased incidence of breast cancer, particularly the early onset breast cancer, in families affected by LFS relative to other forms of cancer has not yet been elucidated (91-93). Taken together, these results strongly suggest that the wild-type p53 gene may function as a suppressor of cellular growth in human breast cancer cells.

P53 is known to regulate cell cycle progression by modulating transcription and by interacting with cell cycle regulatory proteins (94-96). Recent studies have shown that normal p53 protein is a cell cycle checkpoint determinant that controls the length of G1 phase to ensure an intact genome (97,98). Exposure to DNA-damaging agents, such as radiation, leads to an increase in p53 levels

followed by G1 arrest. Cells that lack wild-type p53 protein fail to arrest in G1 following irradiation, and transfection of wild-type p53 restores this response (98-99). These results have led to the hypothesis that p53 is part of a protective mechanism to prevent propagation of DNA damage. Loss of wild-type p53 protein by deletion or mutation may allow for the accumulation of mutations that lead to aberrations in cellular growth control and eventual tumorigenesis. Consistent with this hypothesis, epidemiological studies have shown an increased incidence of breast cancer in younger women who received diagnostic or therapeutic radiation for either breast cancer or other clinical disorders (100-102).

SPECIFIC AIMS.

Ionizing radiation in the form of x-rays is used extensively in the diagnosis and treatment of human breast disease. Although it is known to be a causative agent in breast cancer, the mechanisms by which this occurs remains poorly defined. It has been shown that normal human mammary epithelial cells can be transformed to a malignant phenotype with serial doses of x-rays to a cumulative dose of 30Gy.This malignant transformation involved the introduction of deletions into both alleles of the p53 gene resulting in the loss of expression of the wild-type protein This study shows that the p53 tumor suppressor gene is a target for the mutagenic effects of ionizing radiation. **We propose to test the hypothesis that ionizing radiation transforms immortalized/initiated breast cells from a donor with Li-Fraumeni syndrome (LFS), which contain a germline mutation, and a normal breast cell line containing an acquired p53 mutation to a malignant phenotype. Further, that specific genetic alterations in the remaining wild-type p53 alleles are involved in the development of a malignant phenotype following radiation exposure.**

AIM 1. TRANSFORMATION OF HUMAN BREAST EPITHELIAL CELLS WITH IONIZING RADIATION.

A. Exposure of human breast cells to serial doses of x-rays.
B. Determination of cellular characteristics: morphological changes, growth factor dependency, anchorage-independent growth and tumorigenic potential.
C. Determine the status of tumor suppressor gene p53 using immunoprecipitation analysis of p53 protein. Structural integrity of p53 gene by SSCP analysis and DNA sequencing. Chromosomal analysis of radiation-transformed human breast cells

AIM 2. CHARACTERIZATION OF RADIATION-TRANSFORMED HUMAN BREAST EPITHELIAL CELLS.

A. Verify suppression of tumorigenic growth of transformed breast cells by wild-type p53.
B. Determine potential for radiation-induced G1 arrest in immortalized and transformed breast cells

EXPERIMENTAL DESIGN and METHODOLOGY

AIM 1.Transformation of Human Breast Epithelial Cells with Ionizing Radiation.

Cells and Culture conditions. The recent isolation of an immortalized human breast epithelial cell line from a patient with Li-Fraumeni's syndrome (103), and an additional cell line immortalized by the introduction of a mutant p53 into normal mammary epithelial cells has provided means of testing the previously stated hypothesis. The LFS cell line (HME50) is grown in MEBM medium (Clonetics Corp. San Diego, CA.) supplemented with 0.4% bovine pituitary extract (BPE), 5ug insulin, 10ngs of epidermal growth factor (EGF), 0.5ug hydrocortisone and 5ug transferrin. Epithelial cells grown under these conditions express cytokeratins 14 (a basal cell marker), and cytokeratin 18 (a luminal cell marker). Breast epithelial cells obtained from milk appear to have more of a luminal cell type (expressing cytokeratin 19), as is the case for the majority of breast tumors , with only a subset showing evidence of basal markers (104). At this point in time there are no non-virally immortalized human breast cell lines available which express a keratin profile typical of a luminal phenotype, consequently the use of p53 immortalized human breast epithelial cell lines eg. HME50, that do not strictly express a luminal cytokeratin profile are the only ones available for this type of study. The second cell line we propose to use is a human breast epithelial line immortalized following transfection with an expression vector containing a mutant p53 cDNA (containing a mutation at codon 273). Both of these cell lines do not exhibit anchorage-independent growth and are non-tumorigenic in athymic mice. Finally, normal human breast epithelial cells, containing two wild-type p53 alleles will be used as a control. These cell lines are a generous gift from Dr J Shay of University of Texas, Southwestern Medical Center, Dallas, Texas.

DNA sequence analysis of PCR generated fragments of the p53 gene using primers flanking exon 5 demonstrated the presence of a mutation at codon 133, resulting in an exchange of a methionine for a threonine leading to a conformational change in the wild-type p53 protein (105,106). A similar DNA analysis of the donor's affected relatives indicated that this mutation underlies the high frequency of early onset breast cancer in this family, with the incidence being traced back three generations (103). The conformational change induced by the mutation at codon 133 in this LFS breast cell line results in a loss of DNA binding activity and transcriptional activation by this protein (107). In contrast, the mutation at codon 273 does not result in a conformational change in the p53 protein. This mutant p53 protein retains the ability to bind to the p53 consensus element and its transcriptional activation functions (108). It is entirely possible that these two immortalized human breast cells will differ in their response to x-rays, either in their ability to undergo malignant transformation or radiation-induced cell cycle arrest.

Radiation treatment of human breast cells. Human breast cells will be irradiated using a protocol similar to that used in the therapeutic treatment of breast carcinoma in situ. Exponentially growing cells will be inoculated at 5 x 10^5 per 75cm flask and irradiated 24hrs later with 2Gy of gamma radiation using a JL Shepard Mark II Cesium137 irradiator at a dose rate of 2.37Gy/min. Additional doses of 2Gy will be delivered at daily intervals to a total dose of 60Gy. After each 10Gy increment, the cultures will be allowed to recover for 7-10 days. After this recovery, a portion of the irradiated cultures will be tested for changes in morphology, anchorage-independent growth, growth factor requirements (ie removal of BPE, EGF or insulin), growth in presence of serum and tumorigenicity. The remaining cells will be used for the additional radiation treatments to a total dose of 60Gy.

Assay for anchorage independence. The development of the anchorage-independent phenotype following radiation treatment will be determined by the method of McPherson (109). Potential transformants are suspended at a concentration of $1x10^4$/ml in medium containing 0.3% agar; each sample being plated over a 0.9% agar base layer. Cells will be examined for clonal growth at 21 days; the number of colonies are counted and expressed as the number of cells plated x 100 (colony-forming efficiency).

Tumorigenicity. Three-week old athymic female nude mice (scid) are inoculated in the abdominal mammary fatpad with 10^7 unirradiated human breast cells or radiation-induced transformants or positive control cells MCF7 carcinoma cells. The animals will be examined twice each week for tumor formation. Tumors will then be excised and (i) re-established in culture ; (ii) processed for pathological analysis; and (iii) frozen in nitrogen for later analysis.

STATEMENT OF WORK

The proposed studies are designed to test the hypothesis that ionizing radiation (x-rays) can promote the development of malignant cells from immortalized human breast epithelial cells which contain a mutant p53 tumor suppressor gene. Further, they will determine if the remaining wild-type alleles are targets for the mutagenic effects of ionizing radiation in this type of transformation.

YEAR 1

Radiation treatment of immortalized human breast epithelial cells containing p53 gene mutations with repeated doses of x-rays (2Gy). Cellular characterization of radiation transformed human breast cells: morphology (focus-formation), growth characteristics (growth factor independence), anchorage independent growth (soft agar colony formation),and tumorigenicity (tumor formation in athymic mice). Chromosomal and isoenzyme analysis.

PRELIMINARY STUDIES

The preliminary evidence relating to the investigation of this hypothesis includes: 1.Epidemiological studies have shown that individuals with germline mutations of the p53 gene have an increased incidence of breast cancer (21-23). 2. X-rays have been shown to transform normal human breast epithelial cells to a malignant phenotype (40).This transformation involved the loss of normal p53 protein due to the deletions in both wild-type alleles following radiation exposure. 3. Immortalized human breast epithelial cells from donors with Li-Fraumeni's syndrome, which contain a germline p53 mutation have been established as model systems (103). 4. We have previously shown that multiple doses of x-rays will transform immortalized human keratinocytes to a malignant phenotype (38).

Our prior experience with the human epidermal keratinocyte cell system will provide an important comparison for the proposed studies. The preliminary data we present demonstrates our prior experience and the feasibility of the cellular and molecular approaches we propose to use in the analysis of a radiation-induced malignant phenotype in human breast epithelial cells. These studies were performed to develop a model system for the study of radiation-induced transformation in a human keratinocyte system. The initial cellular studies were successful in transforming immortalized human epidermal keratinocytes to a fully malignant phenotype using ionizing radiation (38). These studies were extended to determine the role of p53 and ras genes in this transformation (48).

To initiate the development of this model system, we used a protocol similar to that of Namba et al (42) as a basis for our transformation studies. Exponentially growing immortalized human epidermal keratinocytes were inoculated a 5 x 10^5 cells per flask and irradiated with graded doses of x-rays, (0,2,4,6, and 8 Gy), at a dose rate of 2.3 Gy min^{-1}. Following irradiation the cultures were allowed to grow to confluence with a change of medium every three days. After exposure of these immortalized human epidermal keratinocytes to single graded fractions of x-rays (0-8 Gy) and growth to confluence, no morphological differences between treated and control cultures could be seen. However, following a second dose of x-rays, foci of morphologically altered cells were observed at confluence in those cultures treated with 2 and 4 Gy fractions. No such foci were observed in either the unirradiated or the 6 and 8 Gy fractions. The longer the 2 and 4 Gy cultures were held at confluence the more defined the foci became. Upon subsequent subculture this type of morphologically altered cells predominated at the expense of the unirradiated immortalized keratinocytes.

These radiation transformed cells were further characterized by quantitative differences in growth properties, such as an increase saturation density and growth in soft agar, both of which are associated with the neoplastic phenotype. The saturation densities of the radiation transformants were between two and three times higher than that of the unirradiated keratinocytes. Additionally, these morphologically altered cells grew in soft agar with colony-forming efficiencies of 0.2 and 0.6 for the 4 and 2Gy fractions, respectively. The unirradiated cell did not efficiently produce colonies of this size when plated in soft agar.

Human breast cells were irradiated using a protocol similar to that used in the therapeutic treatment of breast carcinoma in situ. Exponentially growing cells were inoculated at 5 x 10^5 per 75cm flask and irradiated 24hrs later with 2Gy of gamma radiation using a JL Shepard Mark II Cesium 137 irradiator at a dose rate of 2.37Gy/min. Additional doses of 2Gy were delivered at daily intervals to a total dose of 60Gy. After each 10Gy increment, the cultures were allowed to recover for 7-10 days. After this recovery, a portion of the irradiated cultures were tested for changes in morphology, anchorage-independent growth, growth factor requirements (i.e. removal of BPE, EGF or insulin), growth in presence of serum and tumorigenicity. The remaining cells were used for the additional radiation treatments to a total dose of 60Gy. The development of the anchorage-independent phenotype following radiation treatment. Potential transformants were suspended at a concentration of 1x10^4/ml in medium containing 0.3% agar; each sample being plated over a 0.9% agar base layer and cells were examined for clonal growth at 21 days. Three-week old female SCID mice were inoculated in the abdominal mammary fatpad with 10^7 unirradiated human breast cells or radiation-induced transformants or positive control cells MCF7 carcinoma cells.

We have tested human breast cells isolated from a donor with Li-Fraumeni syndrome and treated with 10,20,30,40,50 and 60 Gy of x-rays for tumor formation and anchorage-independent growth. None of these cultures as yet have produced either of these phenotypic changes. In contrast, morphological transformation and increases in saturation density (3-4 fold) at confluence have been observed in the 20Gy treated cultures, Table1. These changes were transmissible to the 30-60Gy treated cultures. In addition, after treatment with 20 Gy of x-rays the HME50 cells were able to sustain growth in the presence of serum (10% fetal calf serum) and could proliferate in the absence of epidermal growth factor (EGF). All cultures both irradiated and treated (0-60Gy) are still dependent for growth in the presence of bovine pituitary extract (BPE).

TABLE 1

Total dose	Saturation density cells per $cm^2 x 10^{-5}$	Soft agar colony formation	Nude mice with tumors 10^7 cells
None	2.1	< 0.01	0/4
10 Gy	2.2	0.056	0/4
20 Gy	3.2	0.063	0/4
30 Gy	4.6	0.022	0/4
40 Gy	4.8	0.030	0/4
50 Gy	4.9	0.082	0/4
60 Gy	5.4	0.061	0/4

Conclusions and Future Studies.

At the completion of these radiation treatments we have produced changes in cellular morphology , saturation density at confluence and growth factor requirement. The next phase of these studies will be to isolate anchorage-independent phenotypes and tumorigenic clones from these radiation treated human breast cells. Upon isolation these two phenotypes the studies into the role of wild type p53 in the radiation -induced transformation of these immortalized breast epithelial cells can be initiated. The possibilities will include wild type p53 genes being mutated by either deletion resulting in the loss of expression of the p53 protein or that additional point mutations have been introduced arising from a misincorporation during DNA repair. In the event of radiation inducing deletions in the wild type alleles this will result in the mutant form of the p53 predominating resulting in a complete "gain in function." Alternatively, if additional point mutations are introduced into the p53 genes, this will have the effect of increasing the gene dosage for mutant forms of p53. These additional point mutations will indicate an error in a DNA repair mechanism or the development of a state of genetic instability in the transformed cells.

It is also possible that at the conclusion of these studies the integrity and function of the p53 genes in the radiation -transformed breast cells remains unchanged. In this event other genetic mechanisms must be involved either through the activation of a proto-oncogene or the inactivation of a tumor suppressor gene. This will lead to future studies involving functional cloning of activated proto-oncogenes using DNA mediated gene transfer or expression cloning with cDNA libraries from the radiation-transformed breast cells. Alternatively, inactivated tumor suppressor genes involved can be identified using a positional cloning strategy. Both experimental approaches will require the prior evaluation of the chromosomal analysis.

Finally, if these radiation transformation studies performed with doses of x-rays used in the treatment of beast disease are successful in evaluating a role for p53, future studies using doses similar to those used diagnostically eg.(0.006Gy) are feasible.

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